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The effects of microplastic on freshwater *Hydra attenuata* feeding, morphology & reproduction.

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Abstract

Microplastic pollution has been a growing concern in the aquatic environment for several years. The abundance of microplastics in the environment has invariably led them to interact with a variety of different aquatic species. The small size of microplastics may make them bioavailable to a great range of species however, the impact this may have is not fully understood. Much of the research on microplastic pollution has focused on the marine environment and species with little research undertaken in freshwater. Here we examine the effect of microplastics on the freshwater cnidarian, *Hydra attenuata*. This study also describes the development and use of a bioassay to investigate the impact of microplastic on freshwater organisms. *Hydra attenuata* play a vital role in the planktonic make up of slow moving freshwater bodies which they inhabit and are sensitive environmental indicators. *Hydra attenuata* were exposed to polyethylene flakes (< 400 µm) extracted from facewash at different concentrations (Control, 0.01, 0.02, 0.04, 0.08 g mL⁻¹). The ecologically relevant endpoint of feeding was measured by determining the amount of prey consumed (*Artemia salina*) after 30 and 60 min. The amount of microplastics ingested was also recorded at 30 min and 60 min. After which *Hydra attenuata* were transferred to clean media and observed after 3, 24, 48 & 96 hrs with changes in their morphology and reproduction (Hydranth numbers) recorded. The results of this study show that *Hydra attenuata* are capable of ingesting microplastics, with several individuals completely filling their gastric cavities. Significant reductions in feeding rates were observed after 30 min in 0.02 & 0.08 g mL⁻¹ and after 60 min in 0.04 & 0.08 g mL⁻¹ exposures. Exposure to the microplastics caused significant changes to the morphology of *Hydra attenuata*, however these changes were non-lethal. This study demonstrates that freshwater *Hydra attenuata* is capable of ingesting microplastics and that microplastic can significantly impact the feeding of freshwater organisms.

Keywords: Microplastic, *Hydra attenuata*, Feeding, Polyethylene

Capsule: Exposure to microplastic significantly reduced feeding in freshwater *Hydra attenuata* using a novel bioassay

1. Introduction

Plastic pollution in the environment has been well studied for a number of decades ([Azzarello and Van Vleet, 1987](#), [Pruter, 1987](#), [Derraik, 2002](#)). The impact of larger plastic material on birds ([Azzarello and Van Vleet, 1987](#)), marine mammals ([Laist, 1997](#)) and turtles ([Tomás et al., 2002](#)) has been given considerable attention however, in recent years the issue of smaller plastic material known as microplastics has been gaining increasing attention ([Andrady, 2011](#)). Microplastics are pieces of plastic < 5 mm ([Arthur, 2009](#)) and have been found in sediments ([Browne et al., 2011](#), [Eriksen et al., 2013](#)), aquatic water bodies ([Collignon et al., 2012](#), [Lechner et al., 2014](#), [Free et al., 2014](#)) and ingested by a range of species with varying feeding strategies and habitats ([Lusher et al., 2016](#), [Welden and Cowie, 2016a](#)). The study of microplastic pollution has primarily focused on the marine environment with comparatively little research conducted on the freshwater environment, however research indicates that microplastic pollution of the freshwater environment may be as prevalent, as reviewed by ([Eerkes-Medrano et al., 2015](#)).

Sources of microplastic in the freshwater environment include treated effluent from wastewater treatment plants (WWTP), with one plant in Scotland estimated to release up to 65 million microplastics into the freshwater/brackish environment everyday ([Murphy et al., 2016](#)). A number of lakes have been investigated for microplastic pollution ([Eriksen et al., 2013](#), [Imhof et al., 2013](#), [Free et al., 2014](#)). The Great Lakes in North America for example, were found to have an average concentration of 43,157 particles per km⁻² with the most populated lake found to have the highest microplastic count ([Eriksen et al., 2013](#)). Research undertaken on microplastic ingestion by freshwater organisms in natural populations ([Faure et al., 2012](#), [Sanchez et al., 2014](#), [Beginagwa et al., 2016](#)) found 12% of wild gudgeons sampled from French rivers ([Sanchez et al., 2014](#)) and 20% of Nile perch and Nile tilapia purchased in a harbour market in Lake Victoria contained microplastic ([Beginagwa et al., 2016](#)).

Several studies have looked at the potential uptake and effects of microplastics on freshwater organisms in the laboratory, these include invertebrate and vertebrate species ([Rosenkranz et al., 2009](#), [Imhof et al., 2013](#)). [Imhof et al., \(2013\)](#) exposed a range of freshwater invertebrate species to microplastic and found

58 5 freshwater species capable of ingesting microplastic. *Daphnia* exposed to 20 nm and 1000 nm
59 fluorescent polystyrene microspheres were found to uptake the spheres at concentrations of 2 $\mu\text{m L}^{-1}$
60 ([Rosenkranz et al., 2009](#)). When placed in clean water after 4 hrs of exposure, 90% of the 1000 nm
61 microspheres were cleared from the *Daphnia* and only 40% of the 20 nm in the same period. Despite its
62 prevalence in the environment and the growing concern over its potential harmful effects there is
63 currently no standardised bioassay for determining the toxicity of microplastic.

64 In the present study, we describe the development and use of a bioassay to investigate the impact of
65 microplastic on the freshwater cnidarian *Hydra attenuata*. *H. attenuata* inhabits slow moving freshwater
66 bodies where they regulate the planktonic structure through selective feeding of these habitats ([Burnett,](#)
67 [1973](#), [Schwartz et al., 1983](#)) and reproduce asexually by budding every three days provided there is an
68 adequate food supply ([Burnett, 1973](#)). *H. attenuata* is easily cultured and maintained in the laboratory
69 and has been used extensively in toxicological assays as they are sensitive environmental indicators
70 ([Quinn et al., 2008a](#)). The effects of wastewater, pharmaceuticals and heavy metals on *H. attenuata* have
71 all been investigated previously ([Karntanut and Pascoe, 2002](#), [Quinn et al., 2004](#), [Quinn et al., 2008a](#)).
72 The hypothesis being tested in this study is that exposure to microplastic will reduce feeding,
73 morphology and reproduction in the freshwater cnidarian *H. attenuata*. The objectives of this study were
74 to expose *H. attenuata* to various microplastic concentrations, record ingestion of microplastic and prey
75 species and observe changes in morphology and reproduction (hydranth numbers). A modified version
76 of a previously developed protocol ([Quinn et al., 2008a](#)) was used to determine the impact of
77 microplastic exposure on the ecologically relevant endpoints of (i) feeding rates (ii) morphology (based
78 on the Wilby, 1988 scoring system (Supporting Information (SI) Figure 1) and (iii) hydranth number.

80 2. Methods

81 2.1 Test Organism

82 Cultures of *H. attenuata* were sourced from a population in the Environment Canada St-Lawrence
83 Centre (SLC), Montreal, Quebec, which have previously been used in various toxicity studies ([Blaise &](#)
84 [Kusui, 1997](#), [Trottier et al., 1997](#), [Quinn et al., 2007](#)). *H. attenuata* were cultured in glass bowls
85 containing 700 mL of Hydra medium (147 mg L^{-1} $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 110 mg L^{-1} 2-[(2-Hydroxy-1,1-
86 bis(hydroxymethyl)ethyl) amino] ethanesulfonic acid, pH 7) at $18\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ with an 8 hr light (Polylux

XL F58W/840, Made in Hungary, 58 watt fluorescent tube outputting 5200 Lumens) and 16 hr dark photoperiod, following the procedure described by [Trottier et al., \(1997\)](#) and were fed freshly hatched *Artemia salina* daily (used within approximately 3 hrs of hatching). All *H. attenuata* selected for the exposures had a morphological score of 10 per [Wilby \(1988\)](#) scoring system (SI Figure 1). Briefly the scoring systems determines toxicity by measuring drastic changes in morphology by observing the contraction of tentacles and the body and is scored from 10 (healthy, elongated tentacles and body) to 0 (disintegration). Scores of 10 – 6 (sub-lethal signs of toxicity such as shortened and clubbed tentacles) are reversible while scores of 5 and below are irreversible and considered endpoints of lethality.

2.2 Microplastic

A microplastic size class of < 400 µm was chosen as the freshly hatched *A. salina* nauplii that are fed to the *H. attenuata* are < 400 µm in size. Preliminary exposures showed that *H. attenuata* were capable of ingesting polyethylene flakes sourced from a commercially available face wash product which provided a cheap, plentiful and environmentally relevant ([Eriksen et al., 2013](#)) supply of microplastic within the appropriate size range (SI Table 1 & SI Method development). The face wash was passed through a 400 µm sieve to remove larger pieces of microplastic. The microplastics extracted were irregularly shaped, blue and clear in colour and their polymer type was confirmed using Fourier Transform Infrared spectrometry (FTIR). The extracted microplastic were washed 3 times with 70% ethanol, distilled H₂O and Hydra media then dried before the amounts used were weighed. The concentrations used in all the exposures were Control, 0.01, 0.02, 0.04, 0.08 g mL⁻¹.

2.3 Exposures and Endpoints

Two different methods of agitating the microcentrifuge tubes to keep the microplastic in suspension were tested, a shaker (Stuart Shaking Incubator SI500) at 75 rpm used for the 0.5 mL tubes and a mechanical rotator (SI Figure 2, built in the University of the West of Scotland by a laboratory technician using a Parvalux Electronic Motor manufactured in Wallisdown, Bournemouth, England) at 26 rpm used for the 2.0 mL tubes. Making comparisons between the two test was mainly confined to the practicality and usefulness of the separate exposure methodologies implemented. Two separate exposures were carried out in 0.5 and 2.0 mL plastic microcentrifuge tubes (Fisher Scientific). The relevant concentration of microplastic was weighed and placed into each tube, that was then filled with Hydra media and inverted 10 times to ensure the microplastic was homogenously mixed. Healthy

(morphology score 10) individual *H. attenuata* with 2 hydranths were selected from the population and carefully added to each tube (3 individuals per tube) using a pipette with each concentration being undertaken in triplicate. The exposures require that healthy *H. attenuata* (morphology score of 10) with 2 Hydranths are used, however these *H. attenuata* only make up a proportion of the total population. *H. attenuata* can have no Hydranths or more than two hydranths. In order to reduce variables, it is important to use similar *H. attenuata*. This meant there was a limit in the number of *H. attenuata* that met the exposure conditions that could be consistently removed from the population at any one time placing a limit on the number of replicates for each exposure.

The 0.5 mL exposure was repeated and the results combined for a total of 6 replicates for the 0.5 mL and 3 for the 2.0 mL exposure (SI Table 2 & 3). *Artemia salina* cysts were left in aerated water (700 mL ddH₂O, 9.8 g NaCl) at 18 °C ± 2 °C for 48 hrs after which the cysts hatch. The freshly hatched *A. salina* nauplii was washed three times in Hydra media and 10 healthy (swimming) individuals were added to each microcentrifuge tube, care was taken to avoid adding *A. salina* directly onto the *H. attenuata* tentacles. The exposures began when the microcentrifuge tubes were added to the apparatus used to mechanically mix the tubes.

Microplastic ingestion and feeding rates were recorded after 30 min and 60 min, after which they were removed using a pipette, placed in a petri dish with clean Hydra media and observed under a dissection microscope. Feeding rates have been used to determine the effects of contaminants in *H. attenuata* previously (Quinn et al., 2007). Microplastic ingestion was determined by counting the number of polyethylene flakes in the gastric cavity, while feeding rates were determined by counting the number of *A. salina* in the gastric cavity. For the recovery test, *H. attenuata* were transferred from the microcentrifuge tube and placed in a well of a 12 well multi-well plate with 2.0 mL of media with three *H. attenuata* per well. Morphology score and hydranth number were recorded after 3, 24, 48 & 96 hrs. Under normal conditions (fed daily) *H. attenuata* reproduce every 3 days asexually by budding. Hydranths start to form on the main body of the *H. attenuata* and eventually breaks off and forms a separate individual.

2.4 Statistics

Statistical analysis was conducted using R studio version 3.2.2. All data was tested for normality using Shapiro Wilks test for normality and equal variance was tested using Bartlett's test. Differences in the

number of microplastics ingested, feeding rates, morphology & hydranth number were determined using one way ANOVA. Multiple comparison test was carried out using Tukey's post hoc test. Feeding rates, morphological scores and hydranth number were all compared to the control to determine significance while microplastic ingestion was compared to the lowest concentration (0.01 g mL^{-1}) to determine significance. Pearson moment correlations were carried out on microplastic ingestion and feeding rates. IC_{50} values (that inhibits the feeding rate by 50%) were calculated using linear regression analysis.

3. Results

3.1 0.5 mL Microcentrifuge Tube Exposure

3.1.1 Microplastic Ingestion

There was a significant increase in the number of microplastic particles ingested by *H. attenuata* in the highest concentrations compared to the lowest concentration after 30 min ($F(3, 20) = 7.185$, $p = 0.002$) and 60 min ($F(3, 20) = 3.44$, $p = 0.036$) time points (Figure 1A). The mean number of microplastic particles ingested was significantly higher in the 0.04 g mL^{-1} concentration at the 30 min ($p = 0.015$, $n = 2.0 \pm 0.53$) time point and in the 0.08 g mL^{-1} concentration at both the 30 min ($p = 0.007$, $n = 2.2 \pm 0.74$) and 60 min ($p = 0.047$, $n = 2.6 \pm 0.85$) time points (Figure 1A).

3.1.2 Feeding Rates

There was a significant decrease in the feeding rates of *H. attenuata* exposed to microplastic after the 30 min ($F(4, 25) = 5.399$, $p = 0.003$) and 60 min ($F(4, 25) = 5.594$, $p = 0.002$) time points (Figure 1B). The mean number of *A. salina* ingested was significantly lower in the 0.02 g mL^{-1} concentration after 30 min ($p = 0.045$) and the 0.04 g mL^{-1} concentration after 60 min ($p = 0.045$), while the 0.08 g mL^{-1} concentration feeding rates were significantly lower at both the 30 min ($p = 0.002$) and 60 min ($p = 0.001$) time points. There was a significant negative correlation between the amount of *A. salina* ingested and the amount of microplastic ingested at the 30 min ($p = 0.046$) and 60 min ($p = 0.003$) time points. The IC_{50} values calculated for the feeding rates were 0.0361 g mL^{-1} for the 30 min exposure and 0.0350 g mL^{-1} for the 60 min exposure.

3.1.3 Hydra Morphology & Hydranth Numbers

The morphology score of *H. attenuata* was significantly lower in the 30 min 0.08 g mL⁻¹ exposure after 3 hrs ($p = 0.026$), with no other significant differences were observed in the 30 min exposure (Figure 2A). Morphological scores were significantly lower in the 60 min 0.08 g mL⁻¹ exposure after 3 hrs ($p = 0.001$), 24 hrs ($p = 0.001$) and 48 hrs ($p = 0.022$) (Figure 2B). Hydranth numbers were only significantly lower in the 60 min 0.08 g mL⁻¹ exposure after 96 hrs ($p = 0.051$) (Figure 2D).

3.2 2.0 mL Microcentrifuge Tube Exposure

3.2.1 Microplastic Ingestion & Feeding Rates

There was no significant difference in the ingestion of microplastic in either the 30 or 60 min exposures ($p > 0.05$) (Figure 3A). Feeding rates were significantly lower in the 30 min 0.04 g mL⁻¹ ($p = 0.037$) and 0.08 g mL⁻¹ ($p = 0.045$) exposures compared to the control (Figure 3B). No significant differences were observed in the 60 min exposure feeding rates ($p > 0.05$) and no feeding was observed at the 0.04 and 0.08 g mL⁻¹ microplastic concentrations. There was a significant negative correlation between the amount of *A. salina* ingested and the amount of microplastic ingested ($p = 0.001$) in the 30 min exposure, there was no significant correlation in the 60 min exposure ($p = 0.183$).

3.2.2 Hydra Morphology & Hydranth Numbers

The morphological score in the 30 min exposure was only significantly lower in 0.08 g mL⁻¹ after 24 ($p = 0.028$) and 48 ($p = 0.012$) hrs compared to the control (Figure 4A). The morphological score in the 60 min exposure was significantly lower in the 0.08 g mL⁻¹ concentration at all time points ($p < 0.05$) (Figure 4B). Hydranth numbers were significantly lower in the 30 min 0.08 g mL⁻¹ exposure after 48 ($p = 0.036$) and 96 ($p = 0.051$) hrs compared to the control (Figure 4C). While the hydranth number in the 60 min exposure was significantly lower in the 0.08 g mL⁻¹ concentration after 24 ($p = 0.030$), 48 ($p = 0.026$) and 96 ($p = 0.050$) hrs (Figure 4D).

4. Discussion

Over the course of the 2.0 mL exposure it became apparent that the mixing of the microplastic at the higher concentrations was causing physical damage to the *H. attenuata* resulting in morphological impairment observed at these concentrations that invalidated the feeding test for these exposures making

a meaningful comparison between the results difficult and not particularly meaningful. In this study, *H. attenuata* were observed to have significantly reduced feeding in both the 0.5 mL 30 & 60 min exposures (Figure 1B), with feeding rates significantly negatively correlated with microplastic ingestion. Exposure to microplastic has the potential to reduce the health of *H. attenuata* by impacting its ability to feed and limiting the amount of prey consumed. This interaction could have a profound impact in the environment, not only on wild populations of *H. attenuata* but also on their prey species. Feeding is an important and ecologically relevant endpoint as fluctuations in feeding can have major effects on the fitness of individuals and reproduction as well as knock on effects to prey species populations ([Kooijman, & Metz, 1984](#)). These potential community level effects could have significant impacts on the stability of freshwater habitats.

H. attenuata was capable of readily ingesting microplastic (Figure 5), with some individuals completely filling their gastric cavity preventing ingestion of *A. salina*. The ingestion of microplastic may effect an organism in a number of ways, it may cause internal damage to the gastric cavity, a false sense of satiation and impairment of appendages ([Gregory, 2009, Wright et al., 2013a, Gall and Thompson, 2015](#)). Normally it takes *H. attenuata* less than 8 hrs to expel any waste material from their gastric cavity, but in the current study this took considerably longer, between 24 to 48 hrs in some individuals to egest microplastic (SI Figure 3 & 4). These results indicate that when exposed to microplastics *H. attenuata* are expending considerably more time and energy clearing their gastric cavity then under normal conditions. During this time, *H. attenuata* may not be able to feed normally as the gastric cavity is full, potentially further impacting on their health. Microplastics were observed to stick to the tentacles of *H. attenuata* which could potentially impair feeding by restricting its ability to move and capture prey. The ingestion of high numbers of microplastic particles was also observed to cause some *H. attenuata* to become positively buoyant potentially making it difficult to remain attached to the substrate and liable to floating, again potentially impacting on its ability to feed however further research would be needed to determine this.

Previous studies on microplastic and freshwater invertebrates have primarily focused on the uptake of microplastic ([Rosenkranz et al., 2009, Imhof et al., 2013, Blarer & Burkhardt-Holm, 2016](#)). Microplastic ingestion has previously been investigated in the freshwater arthropod, *Gammarus fossarum* exposed to 2680 cm⁻² polyamide fibres for 0.5, 2, 8 and 32 hrs and 60,000 polystyrene beads mL⁻¹ for 24 hrs ([Blarer & Burkhardt-Holm, 2016](#)). *G. fossarum* were found to be capable of ingesting the polyamide fibres after

0.5 hrs, however half the individuals expelled the polyamide fibres after one hour in clean media. After 16 hrs, all polyamide fibres were expelled ([Blarer & Burkhardt-Holm, 2016](#)). The polystyrene beads were also ingested but the amounts were not counted only the presence or absence was reported ([Blarer & Burkhardt-Holm, 2016](#)). The influence of microplastic size on uptake has been observed in *Daphnia* ([Rosenkranz et al., 2009](#)), *Daphnia* exposed to polystyrene beads were shown to uptake 1000 nm beads 40 times higher than 20 nm after 60 min. During preliminary feeding tests uniform microspheres were not ingested by *H. attenuata* (SI Method Development), while the irregularly shaped facewash polyethylene flakes better resembling their prey (*A. salina*) were readily ingested. This indicates the potential influence of the shape and size of the microplastics has on the uptake of microplastics in aquatic biota. *H. attenuata* were also observed to ingest fibres in preliminary studies, this was the result of contamination occurring in the exposures and was not intentional (SI Table 4). However, it is of importance as microplastic fibres can make up a considerable amount of the microplastic pollution entering the environment ([Murphy et al., 2016](#), [Napper & Thompson, 2016](#)). The influence of microplastic morphology is an important factor that needs to be taken into consideration when designing microplastic exposure studies in order for a comprehensive assessment of the risks to be made.

Previous studies have investigated the effects on feeding, fecundity and the retention of microplastic in various invertebrate species. *Arenicola marina* (Lugworms) inhabiting intertidal sediments exposed to 5% unplasticised polyvinylchloride (PVC) by weight displayed significantly reduced feeding activity compared to the control. ([Wright et al., 2013b](#)). Feeding reserves were also reduced by up to 50% and the interval between ingestion and egestion event was 1.5 times longer in exposed worms ([Wright et al., 2013b](#)). The ingestion and egestion of microplastic requires a metabolic cost with no nutritional value being derived from the consumed microplastic. The longer time the microplastic is present in the digestive tract would require greater amounts of energy to process potentially reducing the health of the worm. *H. attenuata* were found to still contain microplastics after 24hr but they were completely egested after 48 hr in the 0.5 mL exposure (SI Figure 3).

The effects of microplastic on a marine copepod *Calanus helgolandicus* were investigated by observing changes in feeding, function and fecundity ([Cole et al., 2015](#)). Copepods exposed to cultured algae and 20 µm polystyrene microspheres at a concentration of 73 beads mL⁻¹ over a 24 hr period were found to have reduced feeding, while prolonged exposures over a 9 day period at a concentration of 65 beads mL⁻¹ resulted in the production of smaller eggs with reduced hatching success ([Cole et al., 2015](#)). The

effects of microplastic on reproduction have also been investigated in the oyster (*Crassostrea gigas*) exposed to 2 and 6 μm polystyrene (PS) microspheres at concentrations of 0.023 mg. L^{-1} (Sussarellu et al., 2016). Female oysters exhibited reduced oocyte numbers and oocyte diameter when exposed to microplastic compared to the controls (Sussarellu et al., 2016), which may have an impact on larvae survival and growth of offspring. While male oysters exposed to microplastic were reported to have a 23% reduction in sperm velocity which has the potential to lower their ability to fertilize the female oocytes (Sussarellu et al., 2016). Although *H. attenuata* reproduction was not affected by the presence of microplastic in the 0.5 mL exposure, reproduction is still an important ecologically relevant endpoint to consider when carrying out microplastic exposures.

Ingested microplastic were capable of being eliminated within the *C. helgolandicus* faecal pellets (Cole et al., 2015) and Pacific oyster faeces with no accumulation in the gut cavity observed (Sussarellu et al., 2016). Microplastic were also effectively removed in pseudofaeces by the clam *Atactodea striata* exposed to polystyrene microgranules between 63 and 250 μm at concentrations of 10 items L^{-1} and $1000 \text{ items L}^{-1}$ (Xu et al., 2016). While microplastic was shown to be retained over a period of months in *Nephrops norvegicus* indicating a prolonged period in which the ingested microplastic could affect the individual (Welden et al., 2016b). *Nephrops norvegicus* were fed 1.5 g squid mantle spiked with 5 polypropylene fibres over an 8 month period (Welden et al., 2016b) and compared to a group fed 1.5 g squid mantle only and a starved group over the same period. The microplastic exposed group exhibited a mean decrease in body mass of -0.0189% per day compared to the fed group 0.0795% . The decrease in body mass may be the result of reduced nutrient up take due to the presence of plastic, this suggest that prolonged exposure to microplastic has the potential to gradually reduce the condition of exposed organisms.

Although an impact on the *H. attenuata* morphology in the 0.5 mL microcentrifuge tubes was observed in the present study (Figure 2A & B), these changes were non-lethal and the *H. attenuata* would be able to recover. The effect of microplastic on freshwater invertebrate morphology has previously been looked at using mud snails exposed to concentrations of various polymers (Imhof and Laforsch, 2016). This study found almost no effect on adult morphology but did show some effect on juvenile development (Imhof and Laforsch, 2016). In the present study hydranth numbers (indicating reproduction) remained unchanged throughout apart from the 0.5 mL 60 min 0.08 g mL^{-1} exposure after 96 hrs (Figure. 3D),

which was significantly lower than the control but did not fall below the number present at the beginning of the exposure.

It is somewhat difficult to compare these results with environmental data as this tends to be presented as microplastic counts rather than by weight ([Lusher et al., 2017](#)). There is also the issue of different sampling methodology resulting in very different microplastic abundance estimates ([Quinn et al., 2017](#)). However, in order to test their impact relatively high concentrations of microplastic were used in this controlled bioassay compared to the quantities measured in most environmental samples. Sampling of the northeast Atlantic has shown there to be 2.46 ± 2.43 particles per m^{-3} ([Lusher et al., 2014](#)), which converts to 0.00000246 particles per mL^{-1} . It is unlikely that these particles numbers would equate closely to what was used in the current study considering the lowest concentration used (0.01 g mL^{-1}) would contain approximately 800 particles. However, organisms located close to sources of microplastic may experience significantly higher microplastic concentrations, for example a Swedish harbor located near a polyethylene production plant reported concentrations of 102,000 plastic particles per m^{-3} or 0.102 particles per mL^{-1} ([Noren 2007](#)). A predicted no effect concentration (PNEC) can be extrapolated by dividing the IC_{50} values by a factor of 1000 ([Jones et al., 2002](#)). If the measured environmental concentration (MEC)/PNEC value is <1 then no further assessment is necessary ([Quinn et al., 2008b](#)), the PNEC values for the 0.5 mL microcentrifuge tube exposure was calculated based on particle numbers at 30 and 60 min (Table 1). Using the environmental concentrations measured by [Lusher et al., \(2014\)](#) and [Noren \(2007\)](#), the MEC/PNEC values extrapolated produce values <1 indicating no further assessment is necessary (Table 1). Although still considerably lower than what was used in the current study, these MEC values demonstrate the great variability in microplastic concentrations in the environment. Both MEC/PNEC particle number values calculated are well below 1 indicating that no further assessment is necessary (Table 1). However, due to the variability in microplastic morphology and polymer composition it is not possible to rule out the potential risk of other microplastics not investigated.

Microplastics also have the potential to act as sink of environmental contaminants resulting in them concentrating on to the surface of the microplastic ([Bakir et al., 2012](#), [Rochman et al., 2013](#)), such as heavy metals ([Brennecke et al., 2016](#)) and persistent organic pollutants ([Frias et al., 2010](#)). These sorbed contaminants may subsequently be released from the microplastic once ingested resulting in toxic effects to the exposed organisms ([Rochman et al., 2013](#), [Koelmans et al., 2013](#), [Koelmans et al., 2014](#)).

Japanese medaka (brackish & freshwater fish) were exposed to virgin low density polyethylene (LDPE) and LDPE left in the marine environment to sorb environmental contaminants over a two month period ([Rochman et al., 2013](#)). Sever glycogen depletion was observed in 74% of marine plastic exposed fish, 46% of virgin plastic fish and 0.5% of control fish. Fatty vacuolation was observed in 47% of marine plastic fish, 29% virgin plastic fish and 21% of control fish. Single cell necrosis was also observed in 11% of marine plastic fish and 0% of the virgin plastic and the control fish ([Rochman et al., 2013](#)). The *H. attenuata* bioassay developed in the present study could potentially be used to assess these microplastic co-contaminants in future studies at environmentally relevant concentrations.

5. Conclusions

The results of this study show that exposure to microplastic can significantly reduce the feeding of freshwater *H. attenuata* and that feeding is significantly negatively correlated with microplastic concentration. Significant changes in *H. attenuata* morphology were observed but these were non-lethal while no change was observed in reproduction. Future studies should examine the effects of various microplastic polymers and types (fibres, beads, flakes...etc.) to better understand the potential effects on exposed organisms. It would also be beneficial to use environmentally relevant concentrations in exposures or to attempt to place the results in an environmentally relevant context. This study adds to the growing body of research on the effects of microplastic on freshwater organisms. As freshwater habitats are already heavily stressed by anthropogenic activity ([Strayer, 2006](#)), it is of considerable importance that emerging contaminants such as microplastic are studied to determine their risk to freshwater biota.

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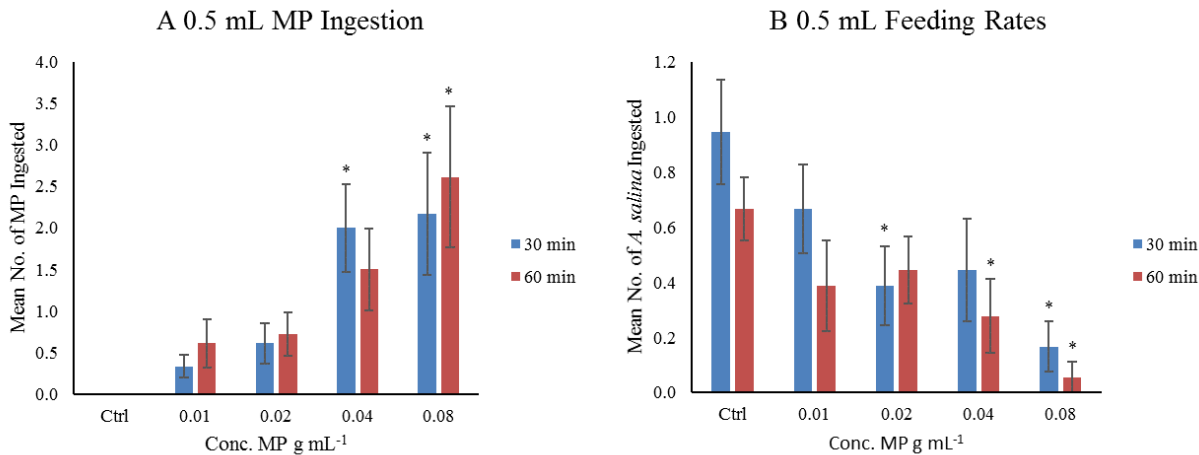


Figure 1. (A) Mean number of microplastics (MP) ingested by *Hydra attenuata* in the 0.5 mL exposure at 30 & 60 min time points (error bars = standard error of the mean, * = significance < 0.05 compared to the lowest concentration of 0.01 g mL⁻¹ determined using Tukey's post hoc test). (B) Mean number of *Artemia salina* ingested by *H. attenuata* in the 0.5 mL exposures at 30 & 60 min time points (* = significance < 0.05 compared to the control).

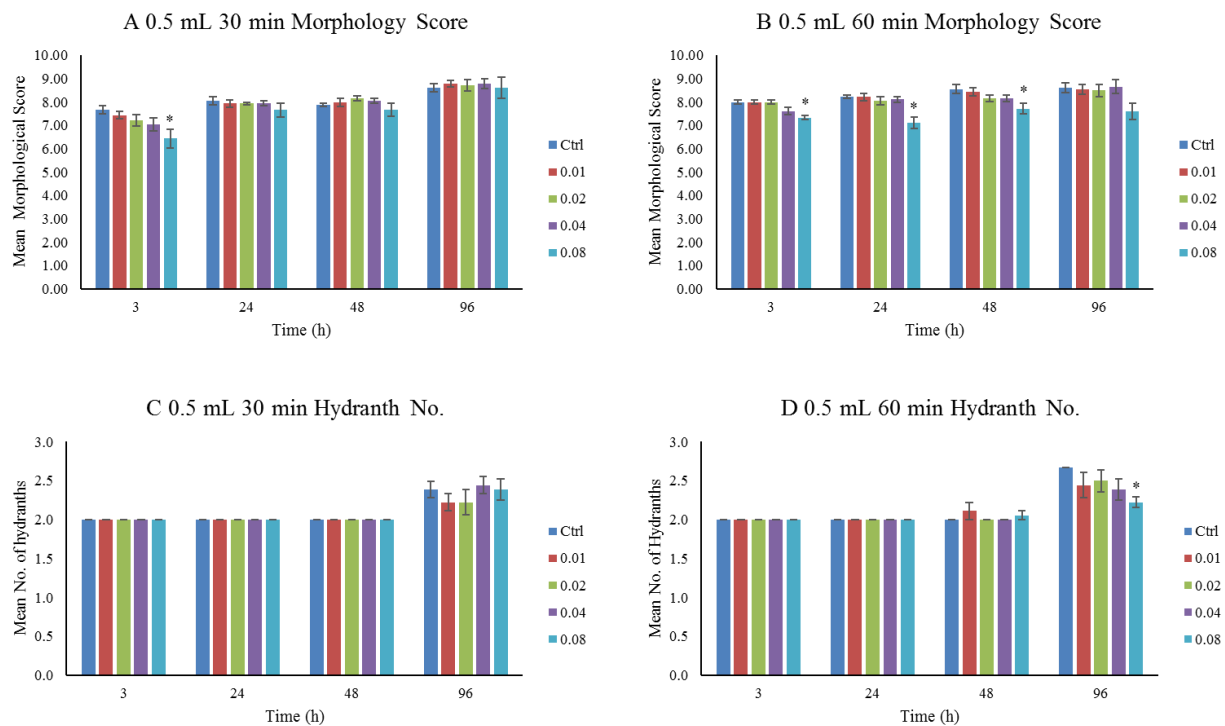


Figure 2. Series of bar charts showing: (A) Mean *Hydra attenuata* morphology scores for the 0.5 mL 30 min exposures at 3, 24, 48 & 96 hour time points (error bars = standard error of the mean,

* = significance < 0.05 compared to the control determined using Tukey's post hoc test): (B) Mean *H. attenuata* morphology scores for the 0.5 mL 60 min exposures at 3, 24, 48 & 96 hour time points: (C) Mean *H. attenuata* hydranth numbers for the 0.5 mL 30 min exposures at 3, 24, 48 & 96 hour time points: (D) Mean *H. attenuata* hydranth numbers for the 0.5 mL 60 min exposures at 3, 24, 48 & 96 hour time points.

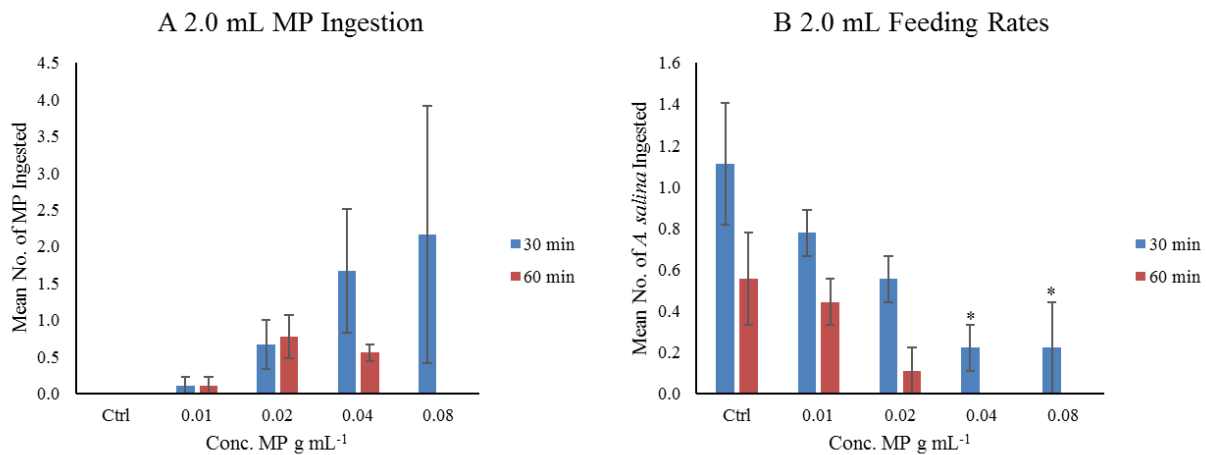


Figure 3. (A) Mean number of microplastics ingested by *H. attenuata* in the 2.0 mL exposure at 30 & 60 min time points (error bars = standard error of the mean, * = significance < 0.05 compared to the lowest concentration of 0.01 g mL⁻¹ determined using Tukey's post hoc test). (B) Mean number of *A. salina* ingested by *H. attenuata* in the 2.0 mL exposures at 30 & 60 min time points (* = significance < 0.05 compared to the control determined using Tukey's post hoc test).

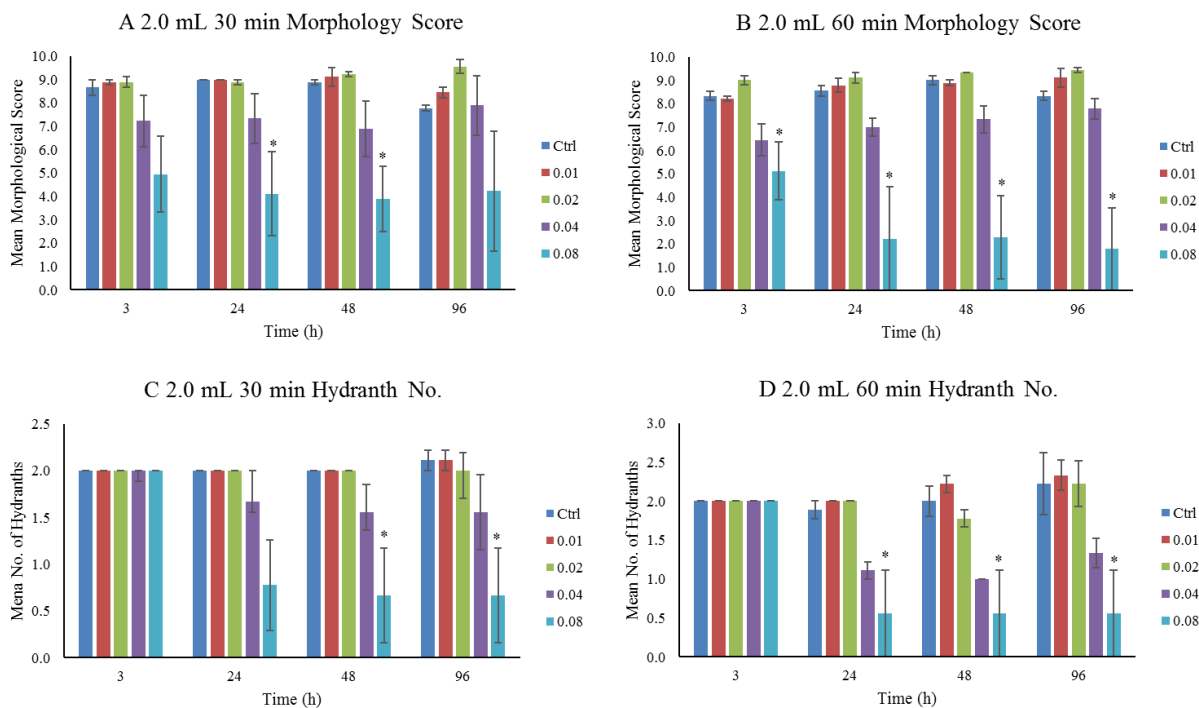


Figure 4. Series of bar charts showing (A) Mean *H. attenuata* morphology scores for the 2.0 mL 30 min exposures at 3, 24, 48 & 96 hour time points (error bars = standard error of the mean, * =

significance < 0.05 compared to the control determined using Tukey's post hoc test): (B) Mean *H. attenuata* morphology scores for the 2.0 mL 60 min exposures at 3, 24, 48 & 96 hour time points: (C) Mean *H. attenuata* hydranth numbers for the 2.0 mL 30 min exposures at 3, 24, 48 & 96 hour time points: (D) Mean *H. attenuata* hydranth numbers for the 2.0 mL 60 min exposures at 3, 24, 48 & 96 hour time points.

Table 1. The measured environmental concentration (MEC), the predicted no effect concentration (PNEC, extrapolated by dividing the Hydra bioassay IC₅₀ by an assessment factor of 1000) and MEC/PNEC values (used for assessment in Tier two toxicity assessment) for microplastics. MEC values were reported by: a = [Lusher et al., \(2014\)](#) & b = [Noren, \(2007\)](#).

Time	No. of Particles per mL ⁻¹		
	MEC	PNEC	MEC/PNEC
30	0.00000246 a	2.888	0.00000085
	0.102 b		0.04
60	0.00000246 a	2.8	0.00000088
	0.102 b		0.04

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568 Figure 5. Photograph of *H. attenuata* with ingested microplastic that can be seen as the blue and
569 transparent particles in the gastric cavity (magnification x25). Due to the buoyancy of the ingested
570 microplastics the foot of this *H. attenuata* was detached from the substrate and the animal was floating.

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Supporting Information

Morphological scores

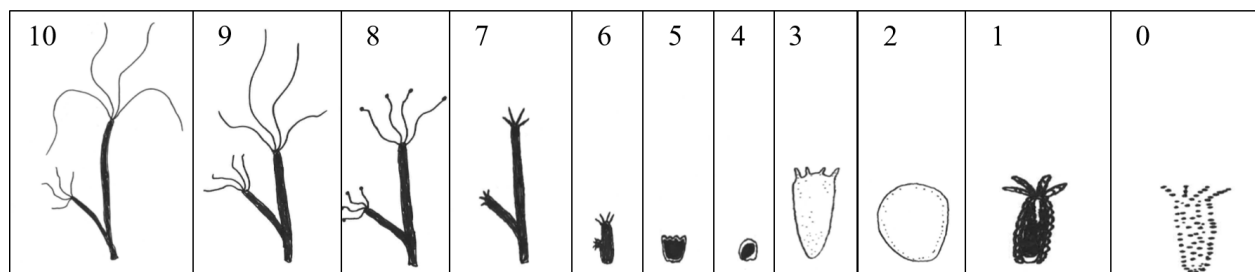


Figure 1. Score 10: Normal. Extended tentacles and body reactive. Score 9: Partially contracted, slow reactions. Score 8: Clubbed tentacles, body slightly shortened. Score 7: Shortened tentacles, body slightly contracted. Score 6: Tentacles and body shortened. Score 5: Totally contracted, tentacles visible. Score 4: Totally contracted, no visible tentacles. Score 3: Loss of osmoregulation. Expanded tentacles visible. Score 2: Loss of osmoregulation. Expanded, no visible tentacles. Score 1: Terminal state, body still intact. Score 0: Terminal state, disintegrated (Based upon [Wilby \(1988\)](#)).

Table 1. Results of first exposure using weighed amounts of facewash microplastic carried out in petri dishes with one *Hydra attenuata* per petri dish and three petri dishes per exposure. Microplastic was mixed manually using the tip of a pipette. The number of microplastic ingested was recorded every 15 min for 120 min.

Time (min)	Concentration (g mL ⁻¹)								
	0.05			0.1			0.2		
15	0	1	0	0	0	1	0	1	0
30	0	0	0	0	0	0	0	0	1
45	0	0	0	0	0	0	1	1	0

60	0	0	0	0	0	0	0	1	0
75	0	0	0	0	0	1	0	1	1
90	0	1	0	0	0	0	0	0	1
105	0	1	1	0	0	0	0	0	0
120	0	0	0	0	0	0	0	1	0

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Figure 2. Photograph of the mechanical rotator used for the 2.0 mL exposures (built in the University of the West of Scotland by a laboratory technician using a Parvalux Electronic Motor manufactured in Wallisdown, Bournemouth, England).

610 Table 2. 0.5 mL exposure mean number of microplastic (MP) & *Artemia salina* ingested and mean morphology score \pm standard error
611 of the mean, n = number of replicates,

0.5 mL Microcentrifuge Tube	Conc. Of MP g mL ⁻¹	n	No. of <i>Hydra</i> <i>attenuata</i>	Mean No. of				Morphology Score							
				MP Ingested		<i>Artemia salina</i> Ingested		3 hrs		24 hrs		48 hrs		96 hrs	
				30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
	0.00 (Control)	6	18	0.0 (± 0.00)	0.0 (± 0.00)	0.9 (± 0.19)	0.7 (± 0.11)	7.7 (± 0.17)	8.0 (± 0.09)	8.1 (± 0.16)	8.2 (± 0.07)	7.9 (± 0.07)	8.6 (± 0.19)	8.6 (± 0.18)	8.6 (± 0.20)
	0.01	6	18	0.3 (± 0.14)	0.6 (± 0.29)	0.7 (± 0.16)	0.4 (± 0.16)	7.4 (± 0.16)	8.0 (± 0.09)	7.9 (± 0.16)	8.2 (± 0.16)	8.0 (± 0.17)	8.4 (± 0.16)	8.8 (± 0.14)	8.6 (± 0.20)
	0.02	6	18	0.6 (± 0.24)	0.7 (± 0.27)	0.4 (± 0.14)	0.4 (± 0.12)	7.2 (± 0.24)	8.0 (± 0.09)	7.9 (± 0.06)	8.1 (± 0.16)	8.2 (± 0.11)	8.2 (± 0.14)	8.7 (± 0.23)	8.5 (± 0.27)
	0.04	6	18	2.0 (± 0.53)	1.5 (± 0.49)	0.4 (± 0.18)	0.3 (± 0.14)	7.1 (± 0.28)	7.6 (± 0.16)	7.9 (± 0.10)	8.1 (± 0.11)	8.1 (± 0.10)	8.2 (± 0.14)	8.8 (± 0.20)	8.7 (± 0.29)
	0.08	6	18	2.2 (± 0.74)	2.6 (± 0.85)	0.2 (± 0.09)	0.1 (± 0.06)	6.4 (± 0.40)	7.3 (± 0.09)	7.7 (± 0.30)	7.1 (± 0.25)	7.7 (± 0.27)	7.7 (± 0.23)	8.6 (± 0.45)	7.6 (± 0.35)

622 Table 3. 2.0 mL exposure mean number of microplastic (MP) & *Artemia salina* ingested and mean morphology score \pm standard error
623 of the mean, n = number of replicates.

2.0 mL Microcentrifuge Tube	Conc. Of MP g mL ⁻¹	n	No. of <i>Hydra attenuata</i>	Mean No. of				Morphology Score							
				MP Ingested		<i>Artemia salina</i> Ingested		3 hrs		24 hrs		48 hrs		96 hrs	
				30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
	0.00 (Control)	3	9	0.0 (± 0.00)	0.0 (± 0.00)	1.1 (± 0.29)	0.6 (± 0.22)	8.7 (± 0.33)	8.3 (± 0.19)	9.0 (± 0.00)	8.6 (± 0.22)	8.9 (± 0.11)	9.0 (± 0.19)	7.8 (± 0.11)	8.3 (± 0.19)
	0.01	3	9	0.1 (± 0.11)	0.1 (± 0.11)	0.8 (± 0.11)	0.4 (± 0.11)	8.9 (± 0.11)	8.2 (± 0.11)	9.0 (± 0.00)	8.8 (± 0.29)	9.1 (± 0.40)	8.9 (± 0.11)	8.4 (± 0.22)	9.1 (± 0.40)
	0.02	3	9	0.7 (± 0.33)	0.8 (± 0.29)	0.6 (± 0.11)	0.1 (± 0.11)	8.9 (± 0.22)	9.0 (± 0.19)	8.9 (± 0.11)	9.1 (± 0.22)	9.2 (± 0.11)	9.3 (± 0.00)	9.6 (± 0.29)	9.4 (± 0.11)
	0.04	3	9	1.7 (± 0.84)	0.6 (± 0.11)	0.2 (± 0.11)	0.0 (± 0.00)	7.2 (± 1.09)	6.4 (± 0.68)	7.3 (± 1.07)	7.0 (± 0.38)	6.9 (± 1.18)	7.3 (± 0.58)	7.9 (± 1.28)	7.8 (± 0.44)
	0.08	3	9	2.2 (± 1.75)	0.0 (± 0.00)	0.2 (± 0.22)	0.0 (± 0.00)	4.9 (± 1.62)	5.1 (± 1.24)	4.1 (± 1.79)	2.2 (± 2.22)	3.9 (± 1.39)	1.8 (± 1.78)	4.2 (± 2.56)	1.8 (± 1.78)

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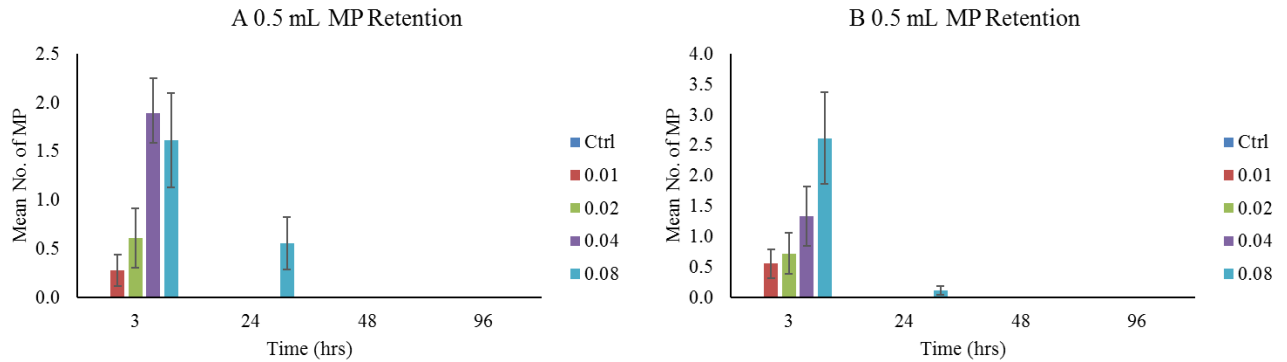


Figure 3. (A) mean number of microplastics retained for the 0.5 mL 30 min exposures at 3, 24, 48 & 96 hour time points (error bars = standard error of the mean) (B) mean number of microplastics retained for the 0.5 mL 60 min exposures at 3, 24, 48 & 96 hour time points.

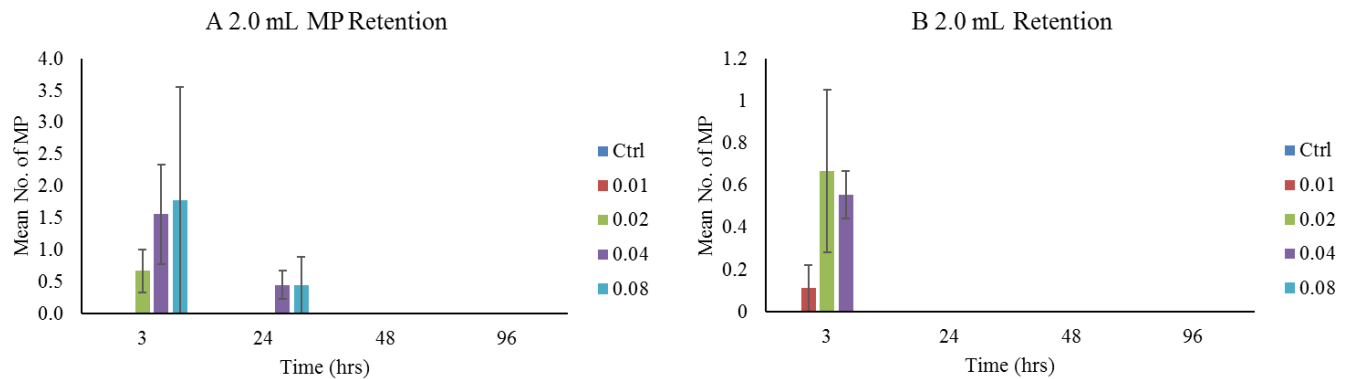


Figure 4. (A) mean number of microplastics retained for the 2.0 mL 30 min exposures at 3, 24, 48 & 96 hour time points (error bars = standard error of the mean) (B) mean number of microplastics retained for the 2.0 mL 60 min exposures at 3, 24, 48 & 96 hour time points.

Method development

The development of a bioassay to assess the effects of microplastic is of great importance in determining what concentrations are of concern to the health of aquatic biota in the environment. *H. attenuata* is a freshwater organism used in standardised tests by organisations such as Environment Canada to test the toxicity of various pollutants (Blaise & Kusui, 1997, Karntanut and Pascoe, 2002, Quinn et al., 2008). This was the primary reason that this species was chosen as the test organism in the present study. As we are attempting to develop a new technique,

several methods of exposing *H. attenuata* to microplastics were investigated before the method used in the present study was finalised. To test the uptake of microplastic by *H. attenuata* an initial exposure involved placing *H. attenuata* in a petri dish with Hydra media spiked with commercially sourced (Cospheric®) florescent polyethylene microspheres. These polyethylene microspheres were within the size range of *H. attenuata* prey (53-63 µm, 180 µm, 425-500 µm) but had a uniform shape and size and were not ingested by the *H. attenuata*. The experiment was repeated using microplastics sourced from a commercially sold facewash product containing irregularly shaped polyethylene flakes which were thought to better resemble *H. attenuata*'s natural prey. One *H. attenuata* was placed in a petri dish. The petri dishes contained different concentration of facewash microplastic (0.05, 0.1, 0.2 g mL⁻¹) and observations were taken every 15 min over 120 min. This exposure proved that *H. attenuata* were capable of ingesting microplastic and these microplastic flakes were subsequently used in all exposures after this (SI Table 1). Although the microplastic was ingested in this set up, the microplastic tended to gather on the side of the dishes the microplastic was therefore not homogenously mixed. Preliminary exposures to microplastics and feeding tests were carried out in petri dishes and 9 well multi-well plates as per the previously published protocol (Trottier et al., 1997). However, as we were using polyethylene with a density lower than the Hydra media (0.926 – 0.940 g cm⁻³) the microplastics did not maintain a homogeneous mixture in suspension and by floating on the surface were physically removed from the test organism.

To allow for a more homogenous mixture the exposure was carried out in 0.5 mL microcentrifuge tubes placed on a shaker which agitated the microplastic sufficiently to keep them in suspension and available to the *H. attenuata* resulting in mixing within the microcentrifuge tubes. In early trials a vortex was to mix the microplastic but was found to be too impractical (Table 4). A mechanical rotator which inverted the 0.5 mL tubes was also tested, but was deemed unsuccessful as little to no mixing of the microplastic was observed. Larger 2.0 mL microcentrifuge tubes were tested using the mechanical rotator and mixing was observed due to the presence of air bubbles. These two methods were then used in the final exposure studies. Over the course of the 2.0 mL exposure it became apparent that the mixing of the microplastic at the higher concentrations was causing physical damage to the *H. attenuata* resulting in morphological impairment observed at these concentrations that invalidated the feeding test for these exposures.

Table 4. Exposure using microplastic flakes extracted from face wash. Each tube contained two or three *Hydra attenuata* and were mixed using a vortex. Contamination resulted in a fibre being ingested by one of the *H. attenuata*. Note: The amount of microplastic used in each tube was roughly the same however this was not weighed but determined by eye.

		Time (min)						
0.5 mL ⁻¹ 1		15	30	45	60	75	90	105
Tube 1	2 Flakes		0	0	0	0	0	0
Tube 2	0	1 Fibre	0	0	0	0	0	0
Tube 3	0	0	0	0	0	0	0	0

Tube 4	0	0	0	0	0	0	0
Tube 5	0	0	0	0	0	0	0

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